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THRESHOLD CHANGE IN EXPRESSION OF GFP-FABD2 FUSION PROTEIN DURING DEVELOPMENT OF *ARABIDOPSIS THALIANA* LEAVES

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One of the most important technical challenges in cell biology is visualization of the actin cytoskeleton. The widely used GFP-FABD2 fusion protein is a helpful tool for investigating actin architecture in living plants. Here we report our attempt to visualize F-actin in mature leaves of transgenic *Arabidopsis thaliana*. With a confocal microscope we observed loss of GFP fluorescence in mature *Arabidopsis* leaves between 19 and 21 days of development. As this pattern was characteristic of all investigated plants and dependent on the age of the plants, we performed precise expression studies at the mRNA (semiquantitative PCR) and protein (Western blot) levels. Our results clearly show a sudden decrease of GFP-FABD2 expression in *Arabidopsis* leaves after the third week of growth. This means that transgenic *Arabidopsis* bearing the *GFP-FABD2* construct is not a good model system for visualization of the actin cytoskeleton in leaves of mature plants.

Key words: Actin, *Arabidopsis thaliana*, gene silencing, GFP-FABD2, mature leaves.

INTRODUCTION

Visualization of actin cytoskeleton architecture in plant cells presents a challenge. Actin filaments may be labeled with phalloidin conjugated to a fluorescent marker (Krzeszowiec et al., 2007), or immunocytochemically with antibodies against actin and fluorescence-labeled secondary antibodies (van Gestel et al., 2001). The main drawback of these methods is that they require cell fixation. The unique features of green fluorescent protein (GFP) enable imaging of cells in vivo. Since efforts to visualize actin conjugated to GFP have remained unsuccessful, an indirect method using fusion constructs bearing GFP and actin-binding proteins has been developed. Among them, the GFP-FABD2 fusion protein derived from the C-terminal part of *Arabidopsis* FIMBRIN1 (Sheanan et al., 2004; Wang et al., 2004; Voigt et al., 2005) is considered one of the best systems. It enables visualization of very detailed F-actin structures in practically all cell types of *Arabidopsis* seedlings (Voigt et al., 2005). Stably transformed *Arabidopsis* does not show phenotypic changes, nor any alteration of phototropic and gravitropic responses (Voigt et al., 2005), unlike GFP mouse talin construct GFP-mTn, which perturbs the diffuse growth of cells (Sheanan et al., 2004). The GFP-mTn system also shows diffuse labeling of the nucleoplasm, a phenomenon not observed in plants expressing GFP-FABD2 (Sheanan et al., 2004). Along with some positive features, the GFP-FABD2 system also has some drawbacks. Actin networks observed in the pollen tubes of *Nicotiana tabacum* transformed with *GFP-FABD2* are differently visualized in fixed cells (Wilsen et al., 2006). Moreover, the presence of this fusion protein reduces cytoplasmic streaming (Holweg, 2006). Finally, we can report here that *Arabidopsis* stably transformed with *GFP-FABD2* shows rapid loss of fluorescence during the growth and maturation of leaves.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS

Arabidopsis thaliana wild-type Columbia was acquired from Lehle seeds (Round Rock, TX, U.S.A.). The seeds of *Arabidopsis* transformed with a *GFP-FABD2* construct were given us by Dr. Boris Voigt (Bonn, Germany). *Arabidopsis thaliana* plants were vernalized for 2 days after sowing and grown in commercial soil in an environmental chamber (Sanyo MLR 350H, Japan) at 23°C and constant 85% humidity, with a 10 h photoperiod. Illumination was

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provided by fluorescent lamps (Sanyo, FL40SS.W/37; light intensity 70–100 μmol m^{-2} s^{-1}).

SEMIQUANTITATIVE PCR

Total RNA was isolated from leaves using Trizol reagent (Invitrogen, U.S.A.) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using a Revertaid™ First Strand cDNA Synthesis Kit (Fermentas UAB, Lithuania) with random hexamer primers. A QuantumRNA™ 18S RNA Kit (Ambion Europe Ltd, UK) was used as internal standard (18S primer : competimer ratio 3:7) in simultaneous PCR amplification with 25 cycles. For amplification of the *GFP-FABD2* construct the primers used were forward 5'CCCGGGATG GTAAAGGAGAAGAACTTTTCACTGGAGTT3' and reverse 5'GCTTGCAGTTCATTCTCTCTGC3'. For amplification of *Arabidopsis FIMBRIN1* the primers were forward 5'ATGTCTGGGTACGTGGGTGT3' and reverse 5'ATCCTTTGCATCTAGTGTAGCCG3'.

WESTERN BLOT ANALYSIS

Leaves were lyophilized overnight, homogenized in liquid nitrogen, suspended in denaturing Laemmli buffer and incubated 10 min at 100°C. Samples were separated by SDS-PAGE on 10% gel and transferred to a PVDF membrane. Detection was performed using an anti-GFP primary antibody (Living Colors GFP Monoclonal Antibody, Clontech, no. 632375, Canada), followed by secondary antimouse antibody conjugated with alkaline phosphatase (Sigma, No. A3562, U.S.A.).

CONFOCAL MICROSCOPY

GFP fluorescence was tested every 3 days starting from the seventh day of growth up to the end of the third week. Seedlings were gently infiltrated with distilled water in a plastic syringe at room temperature. Images, taken with a BioRad MRC 1024 confocal microscope (BioRad, Hercules, CA, U.S.A.), were collected using a 60x (NA 1.4) PlanApo oil-immersion objective mounted on a Nikon microscope. The excitation wavelength was 488 nm (blue light) emitted by a 100 mW argon-ion air-cooled laser (ITL, U.S.A.) used at 10–30% maximum power for imaging. GFP fluorescence was collected in the green channel with a 540 DF30 filter, and chloroplast autofluorescence in the red channel with a 585LP filter.

RESULTS

GFP fluorescence was monitored starting from the first week of plant growth up to the end of the third week, that is, until complete decay of fluorescence. Distinct bundles of actin were seen in the epidermis and mesophyll cells of 11-day-old *Arabidopsis* leaves (Fig. 1a,b). Particularly strong fluorescence was observed in guard cells (Fig. 1a). Fluorescence diminished during plant growth and maturation. Only the cytoskeleton of guard cells remained labeled in 20-day-old leaves (Fig. 1c), but fluorescence in these cells dimmed. We emphasize that in our growth conditions and photoperiod these plants were still very immature, at a very early stage of development. At the end of the fourth week no actin network could be observed; only the inner part of the guard cells showed weak and diffuse fluorescence unrelated to GFP (Fig. 1d).

Semiquantitative PCR with a low cycle number was performed to determine the expression profile of the *GFP-FABD2* construct in transgenic *Arabidopsis thaliana* plants. The primers were designed to anneal to the 5' end of GFP and the 5' end of the FABD2 domain. The results (Fig. 2) clearly show that the amount of *GFP-FABD2* transcript rapidly decreased to a very low level after the third week of growth, as maturation of the *Arabidopsis* leaves proceeded. This expression pattern was matched at the protein level (Fig. 3). On Western blots, sudden loss of the GFP-FABD2 fusion protein (the band corresponding to ~72 kDa of the protein marker) was observed after the third week. Interestingly, the decrease of the *GFP-FABD2* transcript coincided with a decline in the mRNA level of natural *FIMBRIN1* in transgenic *Arabidopsis* leaves (Fig. 4), but this effect was not observed in wild-type plants (Fig. 5). To amplify the natural *FIMBRIN1* from *Arabidopsis*, the primers had been designed to anneal to the 5' end of the gene in order to avoid any amplification of the *GFP-FABD2* construct.

DISCUSSION

Stable expression of the *GFP-FABD2* construct in *Arabidopsis* has been widely described, but all studies have focused on root tissues and seedlings at very early stages of development. Images of trichomes, leaf epidermis and stomata come from young plants 4–7 days old (Sheanan et al., 2004; Voigt et al., 2005), and no data from mature leaves are available. Our results make it plain that *GFP-FABD2* expression rapidly decreases in *Arabidopsis thaliana* after the third week of growth. The loss of GFP fluorescence observed with a confocal microscope and the decrease in the amount of fusion protein were due mainly to reduction of the mRNA level.

It is unlikely that *GFP-FABD2* transcription can be regulated by modulating the activity of the 35S promoter, as the latter is a constitutive promoter (Odell et al., 1985). Reports show that the 35S promoter is more active in young leaves of tobacco (Williamson et

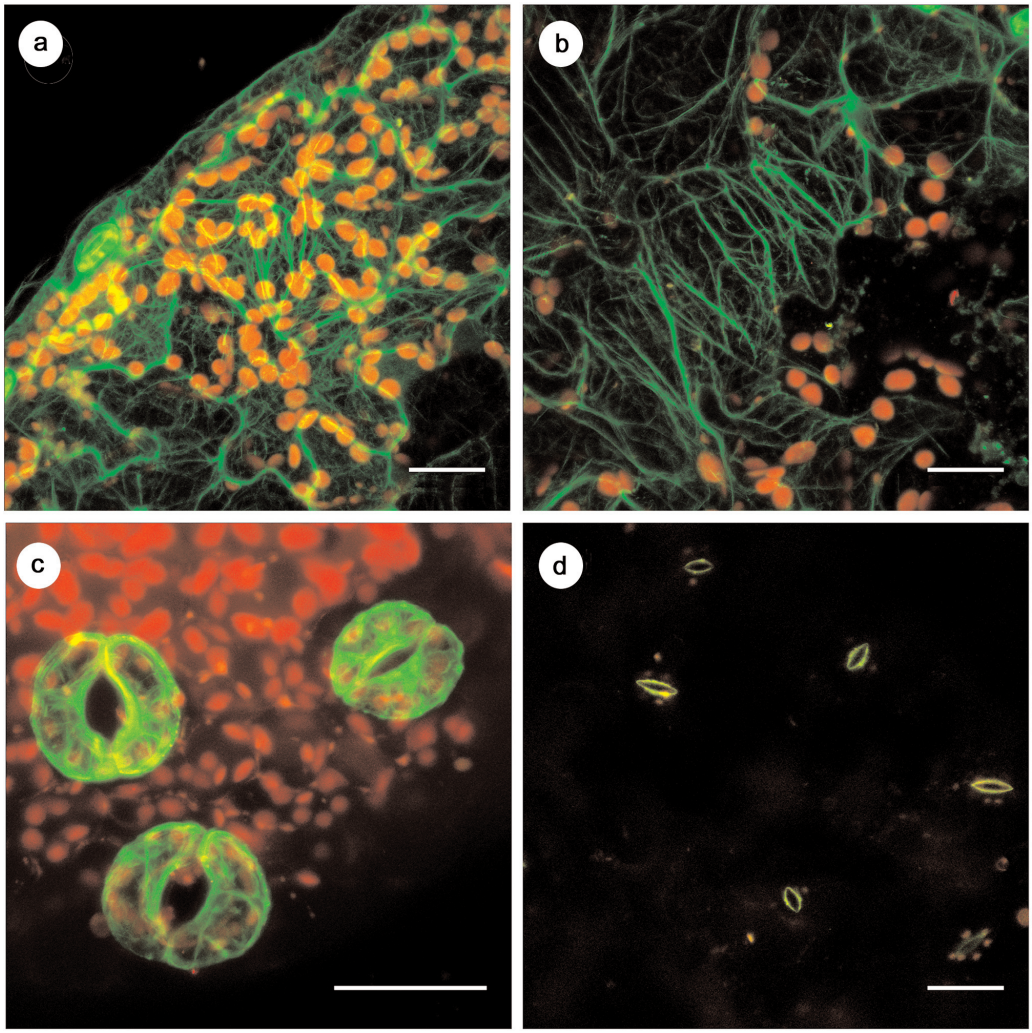

Fig. 1. Actin network in transgenic *Arabidopsis thaliana* bearing *GFP-FABD2* construct. (**a**) Epidermis of young leaf. Guard cells, strong labeling seen at left, day 11 of growth, (**b**) Leaf mesophyll, day 11, (**c**) Guard cells, no labeling of the epidermis is observed, day 20, (**d**) Guard cells, only faint fluorescence visible in inner part, day 27. Bar = 26 μm.

al., 1989) than in old ones, as its expression is S-phase-specific (Nagata et al., 1987). Three-week-old *Arabidopsis* leaves are still developing and actively growing. A more recent study on transgenic cotton transformed with *GFP* under the 35S promoter demonstrates good GFP fluorescence in leaf mesophyll (Sunilkumar et al., 2002). All these data point to RNA silencing as a probable explanation of the effect observed in *Arabidopsis thaliana*.

Cosuppression of an endogenous gene by a transgene has been reported in many plant species (Napoli et al., 1990; de Carvalho et al., 1992; de Borne et al., 1994). Truncated transgenes also trigger this phenomenon (Smith et al., 1990). Two findings of ours support transgene-induced gene silencing in *GFP-FABD2*-expressing *Arabidopsis*. First, loss of natural *FIMBRIN1* transcript was observed along with the decrease in *GFP-FABD2* mRNA. Second, the mRNA level of *FIMBRIN1* remained unchanged in wild-type plants. Rapid loss of the transgene mRNA at some stage of development, with subsequent reset after meiosis, has also been reported in transgenic tobacco (de Borne et al., 1994; Balandin and Castresana, 1997) and *Arabidopsis* (Scheid et al., 1991; de Carvalho et al., 1992; Dehio and Schell, 1994; our observations). A similar pattern of gene silencing, with guard cells unaffected, was shown for tobacco leaves transformed with GFP (Voinnet et al., 1998), and was attributed to the lack of symplastic connections of these cells via plasmodesmata (Wille and Lucas, 1984). Very efficient silencing has been attributed to 35S promotor with a double enhancer (Elmayan and Vaucheret, 1996; Que et al., 1997), the

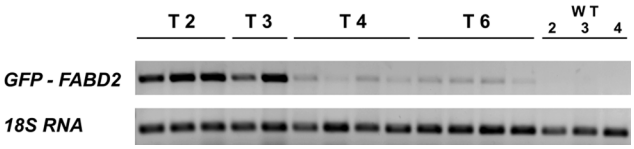

Fig. 2. Expression of *GFP-FABD2* construct at mRNA level in transgenic *Arabidopsis thaliana* (T) and control *Arabidopsis* plants (wild-type, WT): 2, 3, 4, 6 – weeks of growth. 1% agarose gel stained with ethidium bromide.

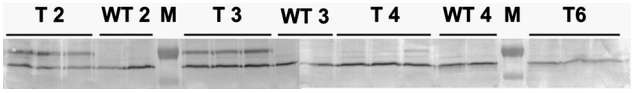

Fig. 3. Expression of *GFP-FABD2* construct at protein level (Western blot) in transgenic (T) and control (wild-type, WT) *Arabidopsis thaliana*: 2, 3, 4, 6 – weeks of growth; M – Page Ruler prestained protein ladder (Fermentas UAB, Lithuania), upper band corresponds to 72 kDa, lower band to 55 kDa. Lower nonspecific band corresponding to ~60 kDa given to show that equal amounts of proteins were loaded per well onto the SDS-PAGE gel.

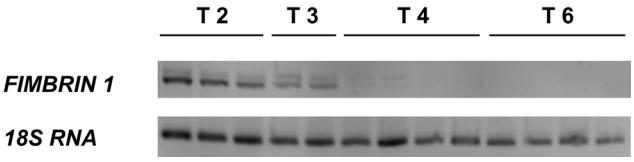

Fig. 4. Expression of *Arabidopsis FIMBRIN1* at mRNA level in transgenic *Arabidopsis thaliana* (T): 2, 3, 4, 6 – weeks of growth. 1% agarose gel stained with ethidium bromide.

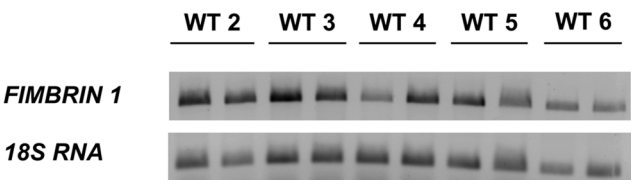

Fig. 5. Expression of *Arabidopsis FIMBRIN1* at mRNA level in *Arabidopsis thaliana* wild-type (WT) plants: 2, 3, 4, 5, 6 – weeks of growth. 1% agarose gel stained with ethidium bromide.

same as that used in the *GFP-FABD2* construct (Voigt et al., 2005). The resulting rise in expression exceeds the putative threshold level beyond which RNA is degraded (Que et al., 1997). This threshold level depends on the nature of the coding region (Schubert et al., 2004). The postulated mechanism by which postranscriptional gene silencing triggered by a sense transgene occurs consists of three steps: conversion of single-stranded RNA to double-stranded RNA by RNA-DEPENDENT RNA POLYMERASE6, cleavage of long dsRNA into siRNA by DICER-LIKE 4, and subsequent sequence-specific ARGONAUTE1-guided mRNA cleavage (reviewed by Vaucheret, 2006). Although this is the most probable scenario, it is worth mentioning that transgene-induced gene silencing of *GFP* under the 35S promotor also has been reported (Schubert et al., 2004), and different classes of siRNA have been detected (Hamilton et al., 2002).

A less likely hypothesis is that the *FABD2* part of the construct is regulated via an endogenous miRNA pathway. Using software developed by Adai et al., 2005, (http://sundarlab.ucdavis.edu/mirna/) we found out that the *FABD* domain of *Arabidopsis FIMBRIN1* has three putative miRNA target sites, but none of the miRNA candidate molecules have been discovered so far.

Interestingly, the loss of fluorescence during maturation of FABD2-based GFP fusion proteins appears to be a general characteristic. It was reported to a lesser extent in *Arabidopsis* plants stably transformed with *GFP-ABD2-GFP* and *ABD2-GFP* constructs (Wang et al., 2008).

Our work shows that the GFP-FABD2 system cannot serve as a model for investigation of the actin cytoskeleton network in mature leaves of *Arabidopsis*. Finding a universal system for F-actin visualization remains a challenge.

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